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CLINICAL STUDY

Human milk oligosaccharide associated with the firmicutes-to-bacteroidetes ratio among stunted infants in Malang, Indonesia

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ABSTRACT

BACKGROUND: Human milk oligosaccharide (HMO) is a unique component of breastmilk. To date, no study has investigated the correlation between HMO and infant nutritional status particularly through the lens of gut microbiota. Therefore, our study aims to investigate the relationships between 2'-Fucosyllactose (2'-FL) in HMO and Firmicutes/Bacteroidetes (F/B) ratio among stunted infants.

METHODS: A case-control study was conducted among 103 mother-infant pairs in Malang City, Indonesia. The quantification of 2'-FL HMO was assessed using High-Performance Liquid Chromatography (HPLC). The F/B ratio was analyzed with real-time poly-chain reaction (RT-PCR). For bivariate analysis, we employed the Spearman correlation and Mann–Whitney tests, while for multivariate analysis, we utilized multiple linear regression.

RESULTS: The findings showed that the stunted nutritional status was detected in 49 out of 103 infants. In this group, 40.81% of mothers of infants with a stunted nutritional status had a secretor-positive status, while all mothers of infants with appropriate nutritional status tested positive for the secretor status (100%). However, the association between maternal secretor status and infant nutritional status was not statistically significant ($p>0.05$). The average levels of 2'-FL HMO in breast milk were lower in the group with stunted infants compared to non-stunted infants (1.21 mg/L vs 1.40 mg/L). The regression analysis revealed a significant association of 2'-FL HMO levels with the presence of Bacteroidetes and value of the F/B ratio ($p>0.05$).

CONCLUSIONS: The breast milk component 2'-FL HMO significantly influences the gut microbiota of stunted infants. Future research aimed at elucidating the mechanisms by which 2'-FL HMO modulates infant gut microbiota should consider not only concentration and specific bacterial taxa but also intake levels *(Tab. 2, Fig. 1, Ref. 37)*. Text in PDF www.elis.sk

KEY WORDS: 2'-fucosyllactose, human milk, oligosaccharide, firmicutes, bacteroidetes, stunting, infant.

Introduction

Stunted growth constitutes 22% of global malnutrition and contributes to half of all child deaths (1). According to Riskesdas 2018 data, the stunting rate among children is 30.8% in Indonesia, ranking the country fifth globally in terms of the number of stunted toddlers (2).

Histopathology involving invasive biopsy of the small intestine is considered the gold standard for diagnosing Environmental Enteric Dysfunction (EED) associated with a dysfunctional gut barrier. However, there is growing interest in developing modified diagnostic markers with reduced invasiveness to identify gut inflammation and heightened intestinal permeability (3, 4). To date, lactulose tests and other blood and urine tests, are the most commonly employed diagnostic methods for identifying EED (5, 6).

In addition to EED, stunting can also result from inadequate infant nutrition (7). Breastfeeding is widely recognized as the gold standard in infant nutrition, particularly during the initial six months of life. Breast milk contains several bioactive components, among which human milk oligosaccharides (HMOs) are the most prominent components after fat and lactose (8).

HMOs play an important role in infant health and nutritional status. Several studies have shown their contribution to developing gut microbiota through the mechanism of selective anti-adhesion properties to pathogenic bacteria (9), facilitating the binding of beneficial bacteria to the intestinal epithelium. Furthermore, HMOs contribute to the lowering of intestinal pH by short-chain fatty acids produced by bacteria, thereby strengthening the function of the intestinal barrier (10). Additionally, HMOs modulate the immune system by restricting the binding of lymphocyte, monocytes, and neutrophils to epithelial cells (11).

However, the number of studies comparing HMOs across Asian countries, particularly those including Indonesia, remains limited (12–15). Given the critical role of HMOs in the health of

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infants, we hypothesize that HMOs may influence the nutritional status of infants in Indonesia, where stunting prevalence remains high. Previous studies investigating interventions to address stunting in Indonesia through nutritional supplementation with multiple micronutrients (MMN), small-quantity lipid-based nutrient supplements (SQ-LNS), or individual micronutrients have yielded inconsistent results (2). These interventions reflect a limited understanding of stunting as solely a form of malnutrition, with solutions primarily focused on feeding practices. Therefore, it is imperative to broaden our understanding of nutritional ecology by considering the absorption process, metabolic pathways involved in digestion and environmental factors (7). This approach is exemplified in studies modulating the gut microbiota of infants, which have shown improvements in the absorption of digestive nutrients and enhancement of their growth (16).

Therefore, this study aims to quantify the concentration of HMO and determine maternal secretor status and its association with infant health status. This research is crucial as it demonstrates that HMOs contribute to infant nutrition, providing a rationale for promoting optimal exclusive breastfeeding practices for managing stunting in Indonesia.

Materials and methods

Study site and participants

This study was conducted among 103 mother-infant pairs in 3 primary health care centers in Malang City, Indonesia, where the prevalence rate of stunted growth remain high. Anthropometric measurements included length-for-age Z-score (LAZ) with a threshold of <–2 standard deviation (SD) indicating stunting. Furthermore, interviews and questionnaires were administered to collect maternal and neonatal data, including maternal age, education, parity, mode of delivery, HMO concentration, secretor status, and stool macroscopic examination. The control group comprised 51 infants with no evidence of stunted growth, while the case group consisted of 51 infants with stunted nutritional status, defined as LAZ <–2 SD.

HPLC was utilized to quantify HMO levels, while macroscopic stool examination was conducted for fecal assessment. The nutritional status was identified as stunted in 49 infants and appropriate in 54 infants. Among the stunted infants, 40.81% had mothers with secretor-positive status (40.81%), while all infants with non-stunted nutritional status had mothers with secretor-positive status (100%). However, no significant association was found between maternal secretor-positive and infant nutritional status ($p>0.05$). Average levels of 2'-FL HMO in breast milk were lower in stunted infants compared to non-stunted infants (1.21 mg/L vs 1.40 mg/L). Analysis using the Mann–Whitney test revealed a significant relationships of 2'-FL HMO levels with infant nutritional status, correlating with the presence of the yellow-colored infant stool, and significant mucus in stool ($p>0.05$). The study highlights the significant role of 2'-FL HMO in infant nutritional status. However, further investigation is warranted to validate the use of macroscopic assessment of stool for detecting inflammation and indigestion in stunted infants.

Breast milk samples collection

Breastmilk expression is conducted at 8–11 AM to minimize variability resulting from the circadian rhythm. Mothers are instructed to manually express milk from the mammary glands, which is then collected in a falcon tube with a 10–15 mL gauge. The expressed breastmilk volume is divided into five 1.5 mL cryovials per 1.5 mL and stored at –80°C in a freezer within twenty-four hours of procurement. The samples are retained until analysis.

2'-FL standard preparation

The commercially available 2'-fucosyllactose (2'-FL) sourced from Sigma Aldrich was prepared by weighing and dissolving 2'-FL in room-temperature water (12 mg/mL). Standard solutions were then prepared by serial dilution, covering a concentration range from 0.2 to 6.6 mg/mL in room-temperature water. The resulting dispersion underwent filtration using a syringe filter with 0.45 μm pores.

2'-FL HMO extraction

The technique for extracting oligosaccharides, as described by Christensen, involves several steps (17). Initially, breast milk is neutralized by thawing at 5°C for 24 hours. Subsequently, aliquots comprising a 1:1 ratio of breast milk and water are prepared. These aliquots are then centrifuged at 10,000 g for 30 min at 4°C, utilizing a Thermo Fisher Scientific Heraus Multifuge X3R centrifuge. The resulting transparent liquid is filtered through a PTFE syringe filter with a 0.25-mm diameter and subjected to further centrifugation at a speed of 7,500 g for 50 min at 4°C. Finally, the filtrate is transferred into an HPLC vial for subsequent analytical procedures.

2'-FL HMO quantification

The quantification of 2'-FL was conducted using the Shimadzu HPLC system equipped with a refractive index detector. HMO separation was achieved by utilizing an Amide column and elution was accomplished by a mobile phase composed of acetonitrile, water, and triethylamine in a v/v/v ratio of 785:215:5. The flow rate during the experiment was set at 0.2 mL/min, with a total runtime of 19 min and the column temperature was maintained at 40°C. The filtrate was introduced to the HPLC equipment through a 2 μL HPLC syringe. Following injection, the syringe was subjected to a rinsing procedure involving a combination of water and isopropanol (900/100, v/v).

Identification of secretor status from breast milk

The determination of secretor status is based on research conducted elsewhere (11). In brief, the oligosaccharide secretor status was determined by measuring structures that bind to α(1,2)-Fuc through activation of the FUT2 enzyme carried by its secretor gene, particularly the 2'-FL gene.

Stool collection

Infant stool samples, in a minimum amount of 200 mg, were collected at home from diapers immediately after defecation. Using a sterile spatula, the samples were transferred into

a sterile stool container and then frozen at -20°C. They were subsequently transferred to a laboratory freezer and stored at –80°C until analysis.

DNA extraction

DNA was extracted using the TIANamp Stool DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) in accordance with the manufacturer's instructions. The extracted DNA was quantified in ng/μL and the purity ratio 260/280 was assessed. The qPCR analysis of 72 samples was conducted using the Rotor Gene kit (QIAGEN) with SYBR Green (Thermo Fischer Scientific, Waltham, MA, USA). The qPCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 15 s and extension at 60°C for 45 s, concluding with a melting curve analysis.

The following primers were utilized for qPCR analysis. For total bacteria, the forward primer 926F (AAA CTC AAA KGA ATT GAC GG) and reverse primer 1062R (CTCACR RCA CGA GCT GAC) were used at a concentration of 0.15 μM. For the Bacteroidetes phylum, the forward primer 798cfbF (CRA ACA GGA TTA GAT ACC CT) and reverse primer cfb967R (GGT AAG GTT CCT CGC GT AT) were used at a concentration of 0.3 μM. For Firmicutes, the forward primer 928F-firm (TGA AAC TYA AGG AAT TGA CG) and reverse primer 1040FirmR (ACC ATG CAC CAC CTG TC) were used at a concentration of 0.25 μM. Prior to usage, these primers were validated in the primer-BLAST database of the National Center for Biotechnology Information (NCBI).

Microtubes with a volume of 20 μL were used for the qPCR setup. Each tube contained 10 μL of SYBR Green, 4 μL of DNA extracted from the samples, and specific primers (denoted as F and R) at following amounts and concentrations. For total bacteria, Bacteroidetes, and firmicutes, 0.6 μL (concentration 0.15 μM), 1.2 μL (concentration 0.3 μM), and 1 μL (concentration 0.25 μM) were set up, respectively. Purified water devoid of DNA and RNA was added to each tube. Additionally, a positive control sourced from DNA extracted from healthy adult feces was included in each run, while a blank containing purified water without DNA or RNA was added for each bacterial phylum. The percentage of each phylum was calculated by interpolating the results from the curve of each bacterial phylum. This interpolation was performed using a determined threshold of 0.05, based on two standard curves generated with a DNA mix comprising 10 samples of infant stool and a known standard for Firmicutes and Bacteroidetes (The ZymoBIOMICS® Microbial Community Standard II (Log Distribution) Catalog # D6310).

Statistical analysis

The characteristics of the respondents were elucidated using percentages and mean values. The relationship of 2'-FL HMO with nutritional status was assessed by categorizing infants into stunted and non-stunted groups. The comparison of 2'-FL HMO levels between stunted and not-stunted infants was conducted using the Mann-Whitney U test. The statistical analysis was performed using SPSS v.26 software (SPSS et al, USA).

Results

Characteristics of respondents

This study analyzed 103 mother–infant pairs, with 49 infants exhibiting stunted nutrition and 54 showing a non-stunted nutritional status.

As shown in Table 1, the comparison of characteristics between these groups indicates that stunted infants are born to younger mothers (28.48 years vs. 30.59 years). Additionally, the group with stunted infants had a lower proportion of mothers with high school education (26.53% vs 74.07%), lower parity (14.2% vs 44.4%) and higher maternal pre-pregnancy BMI. Furthermore, the average level of 2'-FL HMO in breast milk of mothers with stunted infants was lower compared to those with non-stunted infants (1.21 mg/L vs 1.40 mg/L). While the secretor status of maternal milk in the group with non-stunted infants was positive in 100% of cases, it was positive only in 40.81% in the stunted nutritional status group.

Gut microbiota composition differs between stunted and non-stunted infants

A comparison of gut microbiota was conducted between stunted (n=49) and non-stunted (n=54) infants. The research revealed that in the stunted infants, the Firmicutes were more prevalent than Bacteroidetes, whereas in the non-stunted infants, Bacteroidetes predominated over Firmicutes. Consequently, the stunted infants had a higher F/B ratio (Tab. 2), indicative of dysbiosis, an imbalance in gut microbiota composition (22).

Tab. 1. Sociodemographic profile and nutritional status of study participants.

Characteristic	Stunted infants	Non-stunted infants	p
Maternal age (mean + SD)	28.48+5.26	30.59+597	0.316
Graduated from High school (n,%)	26 (26.53)	27 (74.07)	0.107
Parity (mean + SD)	1.78+0.79	2.05±0.95	0.667
Per vaginam delivery (n,%)	14 (14.2)	17 (44.44)	0.695
HMO (mean ± SD)	1.21±0.29	1.40±0.37	NA
Positive secretor status (n,%)	49 (40.81)	54 (100)	0.025*
Stool macroscopic assessment:			
Yellow color (n,%)	32 (65.30)	25 (46.29)	0.137
Loose consistency (n,%)	4 (8.16)	2 (3.70)	0.636
Abundant mucus (n,%)	21 (42.85)	5 (9.25)	0.560

* $p<0.05$

Tab. 2. Comparison of gut microbiota phyla and their ratios between stunted and non -stunted infants.

Phylum	Stunted infants	Non-stunted infants	p*
Firmicutes	219.55 (1425.09)	0.019 (0.054)	0.286
Bacteroidetes	0.69 (1.56)	41.03 (146.40)	0.048
Firmicutes/Bacteroidetes ratio	220656638.6 (1532326800)	221.66 (888.96)	0.319

*Data are shown as means ± standard deviation. Significant differences between groups were calculated using the Pearson test ($p<0.05$)

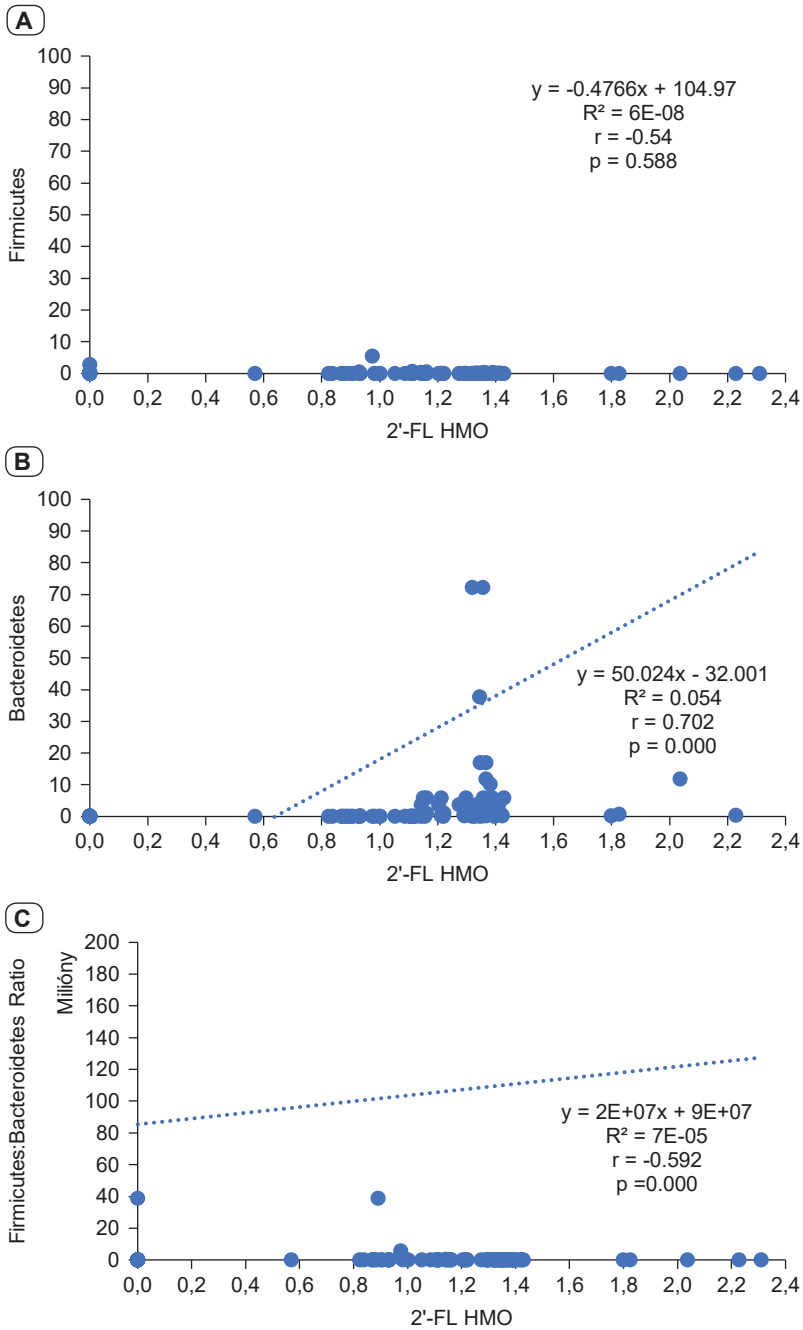

Fig. 1. Correlation of the Relative Abundance of Firmicutes, Bacteroidetes and F/B ratio with 2'-FL HMO. (A) Regression between 2'-FL HMO and the relative abundance of the Firmicutes phylum in infant stool; (B) Regression between 2'-FL HMO and the relative abundance of the Bacteroidetes phylum in infant stool; (C) Regression between 2'-FL HMO and the F/B ratio in infant stool.

Association between 2'-FL HMO and infant gut micobiota

Figure 1 illustrates the association of 2'-FL HMO levels with Firmicutes, Bacteroidetes and the F/B ratio. All regression models demonstrate a statistically significant association of 2'-FL HMO levels with Bacteroidetes and F/B ratio among stunted infants.

It is noteworthy that Bacteroidetes and F/B ratio in infant stool directly correlated with 2'-FL HMO levels. However, when Firmicutes levels were higher, the correlation between Firmicutes and 2'-FL HMO showed an inverse trend.

Discussion

Our study represents the first investigation into the concentration of 2'-FL HMO and infant gut microbiota among stunted infants in Indonesia. We observed an association of 2'-FL HMO and nutritional status in exclusively breastfed infants under six months of age.

HMO stands as the third most abundant solid component of human milk, representing about 20% of human milk carbohydrates (18). Over 200 free oligosaccharide structures have so far been identified in human milk samples (12), with 2′-FL being by far the most abundant, constituting nearly 30% of all HMOs. In fact, these unique complex carbohydrate structures in human milk are virtually absent in cow's or any other farmed animal's milk, with their variety significantly lower (19). However, the findings of various studies examining factors potentially affecting HMOs have been inconsistent (20). These factors often include lactation stages, maternal status, and geographical conditions (21). In our study, we examined the lactation stage in infants aged six months, reflecting the phase of mature breast milk. The average concentration of 2'-FL HMO observed in our study was 1.314 mg/L. This concentration was lower than reported in studies conducted in the USA, Canada, Europe, and China, where concentrations varied in range 4-6 mg/L in breast milk (12). This discrepancy in HMO levels may be attributed by

the HMO analysis method employed in our study which utilized the HPLC method. Studies employing capillary electrophoresis-laser-induced fluorescence (CE-LIF) method in China and other Asian countries yielded notably elevated concentrations, while studies using HPLC reported lower HMO levels (22).

The determination of the secretor status is based on Se (secretor) and Le (Lewis) gene codes, both of which regulate the profile and relative abundance of HMOs expressed by the glucosyltransferase enzyme (FUT) (23). The expression of secretor gene (Se+) leads to abundant production of α1-2-fucosylated HMOs, particularly 2'-FL, whereas the maternal milk from non-secretors, i.e., individuals lacking functional FUT2 gene, contains little to no 2'-FL and other α1-2-fucosylated HMOs due to reduced production of FUT2 enzymes(24). Maternal secretor status was determined by assessing the absolute abundance of the 2'-FL present in each breast milk sample, employing a natural breakpoint in the dataset for accurate classification (24). Based on the cut-off value (1.31 mg/L), 52.42% of mothers were classified as secretors. A similar proportion was also observed in the population of breastfeeding mothers in the Philippines, with only 46% of mothers classified as' secretors (23).

The 2'-FL HMO level observed in this study was lower compared to that found in Europe, America, and Africa, indicating a weak secretor status (20). Deficient secretors are known to produce fucosyltransferase-II (FucT2), an enzyme responsible for synthesizing structures carrying α-1,2-linked fucose residues. However, due to alterations in the amino acid sequence, the effectiveness of this enzyme is considerably compromised. Consequently, the levels of oligosaccharides like 2'-FL in breast milk are significantly below those typically observed in breast milk from secretor mothers (22).

In this study, the stunted nutritional status was not found to be associated with factors such as mother's age, education, parity, or mode of delivery, but it was significantly correlated with levels of HMO, maternal secretor status, and stool macroscopic examination. Recent empirical investigations have established a positive correlation between HMOs and early growth in infancy (11) has been established. Experimental findings suggest that 2'-FL HMOs significantly affect longitudinal development, as evidenced in animal model investigations (20). Additionally, a study conducted in Malawi revealed a positive correlation between the total quantity of HMO present in breast milk and LAZ during the critical developmental phase of infants between 6 and 12 months of age (25).

While the influence of infant human milk intake on breast-fed infant growth is well-documented, the impact of HMO on stunting remains less explored. HMOs play diverse functional roles in infant development (26), serving as prebiotics for beneficial gut bacteria (27), exerting immunomodulatory effects, and selectively reducing colonization of the gut epithelium by certain pathogens, thereby decreasing disease incidence in early life (28).

In this study, the percentages of the bacterial phyla in infant stool showed wide interquartile ranges. Despite the homogenous nature of our study sample, comprising exclusively breastfed infants aged six months from the same geographic region, a significant variability was observed in the distributions of bacterial phyla. This considerable individual variability, although understudied, has already been associated with factors like geographical region, lactation stage, breastfeeding mode and delivery mode (29, 30). Our study provides a window of opportunity to deepen our understanding of infant gut microbiota dynamics.

Notably, this study reveals a significant association between 2'-FL HMO levels and the Firmicutes-to- Bacteroidetes ratio in infant gut microbiota. This ratio has been linked to homeostasis, with alterations potentially leading to various diseases or pathologies, such as obesity and bowel inflammation (31). In our study, the Bacteroidetes phyla were found to be positively correlated with 2'-FL, a prebiotic HMO considered bifidogenic and butyrogenic due to its ability to promote the growth and activity of bifidobacteria and butyrate-producing bacteria via cross-feeding mechanisms (32). Previous research has demonstrated that butyrate can significantly enhance the transepithelial electrical resistance and induce the expression of tight junctions. Furthermore, stimulating the growth of bifidobacteria through HMOs, but not lactose, has been shown to enhance the expression of tight-junction proteins (33).

In contrast, our study showed that 2'-FL HMOs have no correlation with Firmicutes. This lack of correlation may be attributed to the fact that Firmicutes are capable of degrading starch or polysaccharides, as well as other non-digestible dietary nutrient sources such as pectin and cellulose. However, the abundance of Firmicutes was observed to be higher in the stunting group compared to the non-stunted group. Firmicutes can produce more energy than Bacteroidetes from nutrients fermentation products, primarily short-chain fatty acids (SCFAs), particularly butyrate (31). Butyrate serves as an energy source for colonic epithelium, enhancing insulin sensitivity in mice, exhibits anti-inflammatory properties in humans, protects against colon carcinoma, regulates gene expression of leptin, and provides protection against diet-induced obesity. Acetate also contributes to gluconeogenesis, along with propionate. A high abundance of Firmicutes is associated with an increase in energy extraction, leading to copious energy intake (34). On the other hand, the phylum Bacteroidetes predominantly produces acetate and propionate. The concentrations of all individual forms of SCFA, including their total value were higher in feces of stunted children, indicating increased energy-loss though fecal excretion. Moreover, the two short-chain fatty acids propionate and butyrate, were found to be significantly higher in the plasma of stunted children compared to the healthy group (35).

In our study, we observed a higher F/B ratio in the stunted infants compared to those with non-stunted growth. Bacteroidetes possess a vast array of genes involved in polysaccharide acquisition and metabolism, allowing them to easily adapt to diverse environmental niches (36). This adaptability of Bacteroidetes is facilitated by their genomic plasticity, manifesting as continuous genetic rearrangements, duplications, and lateral gene transfers between species (37). However a lower proportion of Bacteroidetes in the stunted infants may indicate deficiencies in multiple microbial pathways, including the N-glycan pathway. This deficiency may lead to less efficient energy extraction from non-digestible polysaccharides or dietary fiber (29).

This study provides primary observational data exclusively from breastfed 6-month-old infants, ensuring homogeneity of the respondent groups. Beyond the primary findings highlighting the impact of 2'-FL HMO concentration on the nutritional status of infants, auxiliary findings reveal an association between 2'-FL HMO and infant gut microbiota. One limitation of this study stems from the lack of total HMO quantification, which precludes the calculation of the proportion of 2'-FL in total HMOs. Nevertheless, in this study, the 2'-FL values were measured absolutely and were associated with specific nutritional statuses in stunted and non-stunted infants. Future research should aim to prospectively assess total HMOs to gain a clearer understanding of their relationship with linear infant growth.

Conclusion

The concentration of 2'-FL HMO is associated with infant gut microbiota, as evidenced by the Firmicutes-to-Bacteroidetes ratio in infant stools. Further analysis is needed to optimize the potential role of HMOs in preventing and treating stunting in exclusively breastfed infants.

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